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論　文　名	Studies on Bioactive Cyclic Peptides Produced by Ascomycete OK-128 (子嚢菌 OK-128 株の生産する生理活性環状ペプチドに関する研究)
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論文要旨

In search of novel compounds for pharmaceutical and agricultural uses, fungi have been subjects of intense studies for their wide array of metabolites. We have previously found that several fungal strains isolated from soil produced interesting secondary metabolites which are active against the silkworm (*Bombyx mori*). *Penicillium simplicissimum* ATCC 90288 produced the insecticidal indole alkaloids, okaramines, and *Aspergillus japonicas* ATCC 204480 produced the paralytic alkaloids, asperparalines. Okaramine B and asperparaline A particularly showed prominent activity against several insect pests, and therefore their mode of action has attracted strong attention. In the course of our continuing search for novel bioactive compounds from fungal metabolites, the methanol extracts of fungi were screening for their insecticidal, paralytic, and convulsive activities using silkworm. This study deals with the isolation of novel active principles, their chemical structures, and bioactivities.

A total of 500 methanol extracts from fungi were screened for their insecticidal, paralytic, and convulsive activities against the third instar larvae of silkworm. As a result, 29 samples showed insecticidal activity in the range of 20-100%, 8 samples paralytic activity in the range of 80-100%, and 40 samples convulsive activity in the range of 20-100%. Because OK-128 showed a significant paralytic activity, the

methanol extract of OK-128 was selected for further investigation of bioactive compounds.

Twenty kg of okara fermented with the fungus was soaked in MeOH for 7 days. The extract was evaporated to aqueous concentrate and the concentrate was extracted with EtOAc. The extracted material (33.2 g) was chromatographed on silica gel by n-hexane and an increasing ratio of EtOAc. The active 70% and 100% EtOAc eluates (22 g) were chromatographed further on silica gel using n-hexane−acetone (75:25) and were crystallized from MeOH to obtain compound **1** (8.8 g). The filtrate was further purified by HPLC to yield three minor compounds: compound **2** (49.6 mg, tR 49.6 min), compound **3** (3.4 mg, tR 86.3 min) and compound **4** (2.0 mg, tR 96.0 min).

Compound **1** and compound **2** were identified as PF1171A and PF1171C, respectively, by comparing their MS, ^{1}H NMR and ^{13}C NMR data with those previously reported. However, the absolute configurations of the residues of the amino acids in PF1171s were not determined in the previous study. The absolute configurations of PF1171A and PF1171C were therefore determined by using Marfey's methodology in this study. The hydrolysates were treated with Marfey's reagent, 5-fluoro-2,4-dinitrophenyl-L-alanine amide (FDAA), and the resulting derivatives were analyzed by reverse-phase HPLC. The peaks in the chromatogram were identified by comparing the retention times with those of the FDAA derivatives of the authentic amino acids. The FDAA derivatives of the amino acids liberated from PF1171A (**1**) showed peaks matching L-Ala, L-Pip, L-Leu, L-MeLeu, and D-Ile. The absolute configuration of the amino acids in PF1171C (**2**) were determined to be L-Ala, L-Pip, D-Val, L-Leu, and L-MeLeu.

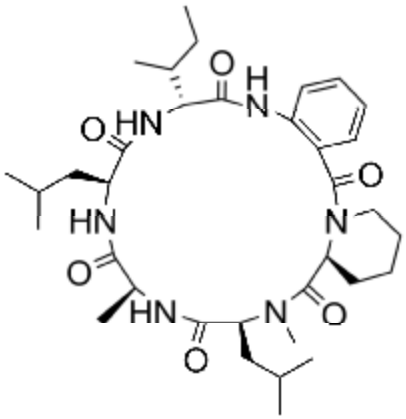
Compound **3** was isolated as a colorless amorphous solid, $[\alpha]_D^{28}$ +18° (*c* 0.15, $CHCl_3$). The molecular formula was determined to be $C_{38}H_{52}N_6O_6$ by the HRFABMS data (*m/z* 689.4020 $[M+H]^+$, Δ −0.7 mmu), requiring 16 degrees of unsaturation. The ^{1}H and ^{13}C NMR spectra of **3** displayed signals from six amide carbonyl and four amide protons, indicating that compound **3** was a hexa-peptide containing two amino acid residues that did not have amide proton. ^{1}H–^{1}H COSY experiment constructed the six amino acid residues as Ile, AA, pipecolinic acid (Pip), Leu, Ala, and Phe. The carbonyl carbons of the respective amino acid residue were deduced from the HMBC correlations of amido, α-, and β-protons. The NH of Leu residue was revealed to be methylated according to the HMBC correlation from singlet methyl singal (δ_H 3.23) to the α-carbon (δ_C 65.3). These residues accounted for 15 degrees of unsaturation, indicating that **3** is a cyclic peptide. The HMBC experiment revealed the following connections of the amino acid residues: AA to Ile, Pip to AA, MeLeu to Pip, Ala to MeLeu, and Ile to Phe. Finally, the planar structure of **3** was determined to be cyclo(Ile-AA-Pip-*N*-methylleucine

(MeLeu)-Ala-Phe). Therefore, based on current data, **3** has been determined to be novel cyclic hexa-paptide belonging to the PF1171s and hereafter is referred to PF1171F.

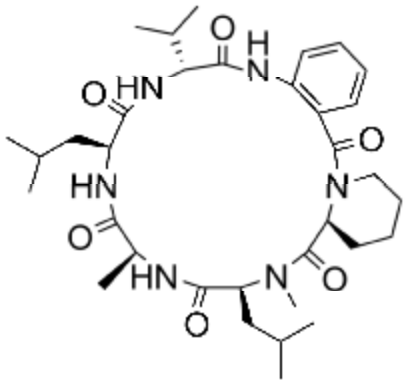
Compound **4** was isolated as a colorless amorphous solid, $[\alpha]_D^{28}$ +26° (*c* 0.15, $CHCl_3$). The molecular formula was determined to be $C_{36}H_{56}N_6O_6$ by the HRFABMS data (*m/z* 669.4344 $[M+H]^+$, Δ +0.5 mmu). The ^1H and ^{13}C NMR spectra were similar to those of PF1171A (**1**). However, the signals for Ala residue observed in the spectra of **1** were not detected in **4**. Alternatively, the signals for methyl (δ_H 0.72, δ_C 9.7), methylene (δ_H 1.68, 1.90, δ_C 24.5), and methine (δ_H 4.78, δ_C 52.9) groups were observed in the spectra of **4**. In addition, the methylene protons (δ_H 1.68, 1.90) were coupled to the methyl protons (δ_H 0.72) and the α-methine proton (δ_H 4.78). These data indicated the presence of a 2-aminobutyric acid (Aba) residue in **4**, which was also confirmed by the latter Marfey amino acid analysis. Other amino acid residues were assigned to be Ile, AA, Pip, MeLeu, and Leu by $^1H-^1H$ COSY and HMBC experiments. Consequently, the structure of a new haxa-paptide **4** was established to be
cyclo(Ile-AA-Pip-MeLeu-Aba-Leu), and was named as PF1171G.

The absolute configurations of the amino acids in PF1171F and PF1171G were determined by Marfey's methodology. The absolute configuration of the amino acids in PF1171F (**3**) showed peaks matching D-Ala, L-Pip, L-Phe, L-MeLeu, and D-Ile. PF1171G (**4**) were determined to be D-Aba, L-Pip, L-Leu, L-MeLeu, and D-Ile. The paralytic activities of PF1171s were evaluated against the silkworm. The paralytic activities of PF1171A (**1**), PF1171C (**2**), PF1171 F (**3**) and PF1171G (**4**) in a dose of 30 μg/g diet were 40%, 16%, 43% and 80%, respectively, against silkworms observed 5 hours after feeding.

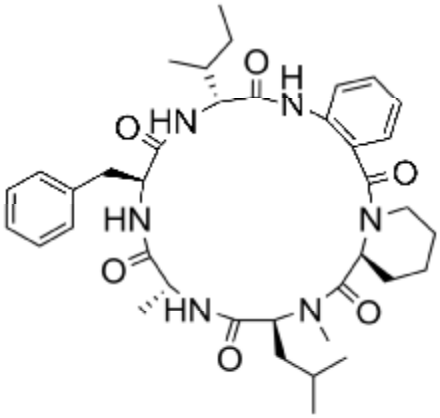
So far, various active cyclic peptides have been shown to act on insects. Among them, paralytic peptide destruxins produced by *Metarhizium anisopliae* are well-studied, and their mechanism of action is suggested to be caused by interfering calcium channels in muscle of *Drosophila melanogaster*. The paralytic response of silkworm to PF1171s is very similar to that to paralytic alkaloid asperparaline A, a blocker of insect nicotinic acetylcholine receptor, suggesting that the peptides also target the neuron systems of silkworm. We are now developing the method to explore the mode of action of insect-active fungal metabolites. The mechanisms of silkworm paralysis by PF1171s will be resolved in near future.



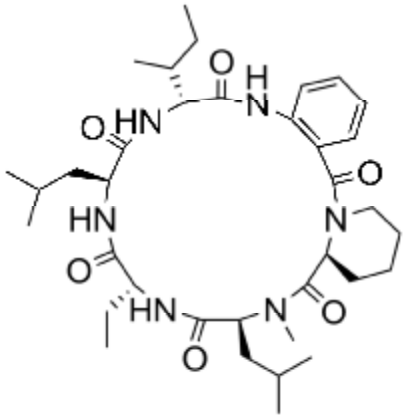
PF1171A



PF1171C



PF1171F



PF1171G

審査結果の要旨

微生物は生理活性物質の宝庫であり，特異な構造を有する種々の化合物が微生物の二次代謝産物として明らかにされてきている．現在も医薬あるいは農薬として有効な化合物の探索が微生物を対象に広く行われている．本申請者は土壌微生物，特に真菌類を対象に害虫防除に有効な物質あるいは新たな薬剤の開発におけるリード化合物となる物質を見出すことを目的として研究を行い，以下の成果を得た．

まず，土壌から真菌を定法により分離した．得られた分離菌株を，オカラを用いて培養した後，メタノールで抽出した．このメタノール抽出物を含んだ人工飼料をカイコに与え，それらの示す活性を精査した．分離菌株 500 株の中で 29 株が殺虫活性を示した．また，8 菌株が麻痺活性を示し、40 菌株が痙攣活性を示した．そこで，本研究で

は顕著な麻痺活性を示した子嚢菌 OK-128 株の生産する活性物質の化学構造の解明を行った.

子嚢菌 OK-128 株のオカラ培養物から得られたメタノール抽出物をヘキサンおよび酢酸エチルで順次抽出した. 活性の認められた酢酸エチル抽出物をシリカゲルカラムクロマトグラフィー（ヘキサン･酢酸エチル混合溶媒系、ヘキサン･アセトン混合溶媒系）を用いて順次精製し, 活性を示す 25% アセトン溶出画分を得た. メタノールを結晶溶媒として用いて本画分から活性物質 1 を得た. さらに, ろ液を HPLC で分取することにより, 活性物質 2 および 3, 4 を得た.

各種スペクトルデータの解析から, 活性物質 1 および 2 はいずれも anthranilic acid (AA) と pipecolinic acid (Pip) を含む環状ペプチドであり, それぞれ PF1171A および PF1171C と同定した. PF1171A と PF1171C は平面構造が報告されているのみであり, 構成アミノ酸の立体化学は不明であった. そこで, Marfey 法を用いて各アミノ酸の絶対立体配置を決定し, 活性物質 1 および 2 をそれぞれ cyclo(L-Ala-L-Leu-D-Ile-AA-L-Pip-L-*N*-MeLeu) および cyclo(L-Ala-L-Leu-D-Val-AA-L-Pip-L-*N*-MeLeu) であると決定した.

活性物質 3 の分子式は HR-FABMS 分析から $C_{38}H_{52}N_6O_6$ であると決定された. ^{1}H-および ^{13}C-NMR スペクトルから 6 個のカルボニル炭素と 2 個のベンゼン環の存在が示唆され, 不飽和度を考慮することにより活性物質 3 が 6 個のアミノ酸から成る環状ペプチドであることが明らかとなった. 二次元 NMR スペクトルデータの解析とアミノ酸分析から活性物質 3 の平面構造を cyclo(Ala-Phe-Ile-AA-Pip-*N*-MeLeu) と決定した. Marfey 法により構成アミノ酸の絶対立体配置は D-Ala および L-Phe, D-Ile, L-Pip, L-*N*-MeLeu であることが明らかとなり, 活性物質 3 の構造を cyclo(D-Ala-L-Phe-D-Ile-AA-L-Pip-L-*N*-MeLeu) と決定した. 本化合物は PF1171 類に属する新規化合物であったので PF1171F と命名した. 一方, 活性物質 4 の分子式は $C_{36}H_{56}N_6O_6$ と決定され, その構成アミノ酸として PF1171 類には前例がないとともに, ペプチド構成成分としても非常に特異な 2-aminobutyric acid (Aba) を含んでいることが明らかとなった. 活性物質 3 と同様にして活性物質 4 の全構造を cyclo(D-Aba-L-Leu-D-Ile-AA-L-Pip-L-*N*-MeLeu) と決定し, 新規化合物であったので PF1171G と命名した.

活性物質 1 および 2, 3, 4 のカイコに対する活性を調べた. すなわち, 各化合物 30 μg を餌 1 g に添加した後これを 3 齢カイコに投与し, 5 時間後の麻痺活性を測定したところ, 1 および 2, 3, 4 はそれぞれ 40% および 16%, 43%, 80% の麻痺活性

を示した．興味あることに非タンパク性アミノ酸である Aba を含む 4 が最も強い麻痺活性を示した．

以上のように，子嚢菌 OK-128 株から麻痺活性を有する 4 種の環状ペプチドを明らかにした．また，カイコを用いる簡便な生物検定系が生理活性物質を検索する際の指標として今なお用い得ることが示された．これらの成果は生物有機化学および微生物化学，昆虫生理学等の分野に多大の貢献をするものと考えられ，最終試験の結果と併せて，博士（応用生命科学）の学位を授与することを適当と認める．